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TITLE: Mechanisms of Graft-vs.-Leukemia Against a Novel Murine Model of Chronic
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ABSTRACT

Our objective is to understand the immunobiology underlying the differential sensitivity of chronic phase and blast crisis CML. Our data thus far support the hypothesis that GVL against mCP-CML can be mediated by redundant processes, and that impairment of an individual pathway is insufficient to prevent GVL. We hypothesize that GVL against BC-CML is less forgiving than that against CP-CML, and that multiple effector pathways must act in concert for effective GVL. In the last year we have: 1) created multiple gene deficient mBC-CMLs; 2) cloned these and established clonality by southern blot analysis; 3) assessed whether host APCs are required for CD4 and CD8-mediated GVL; 4) determined that cognate T cell:leukemia interactions are required for CD4 and CD8-mediated GVL; 5) determined that mBC-CML cells express high levels of B7-H1; 6) obtained B7-H1 gene deficient mice to create B7-H1^{-/-} mBC-CML; and 7) begun a new collaboration to study the role of TGF- β in mBC-CML GVL resistance.

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I. Introduction.

In this second year of funding we have made substantial progress in all tasks.

II. Body.

A. Aim 1 tasks.

1. Establish murine blast crisis (mBC-CML).

- Generate appropriate retrovirus.
- Infect B6 progenitors and transplant into B6 mice
- Phenotype leukemia
- Secondary transplants

All of these were accomplished in the first year of funding.

2. Establish GVL against B6 mBC-CML

Accomplished during the first year of funding.

3. Determine the roles of recipient APCs in CD8 GVL.

To do so we used B6 $\beta 2M^{-/-}$ hosts as recipients in GVL experiments using C3H.SW (H-2^b) mice as donors. As shown in Figure 1., surprisingly, GVL was intact in $\beta 2M^{-/-}$ CD8 recipients ($P < 0.0001$) and survival between $\beta 2M^{-/-}$ and wild type B6 recipients were not significantly different ($P = 0.4113$). One possible caveat to this experiment is that the BC-CML cells may have been contaminated with host-type antigen presenting cells. We have since sorted B6 mBC-CML cells to $>99\%$ purity and demonstrated that these cells are as lethal as the unsorted cells (not shown). We will be using this leukemia shortly in a repeat experiment.

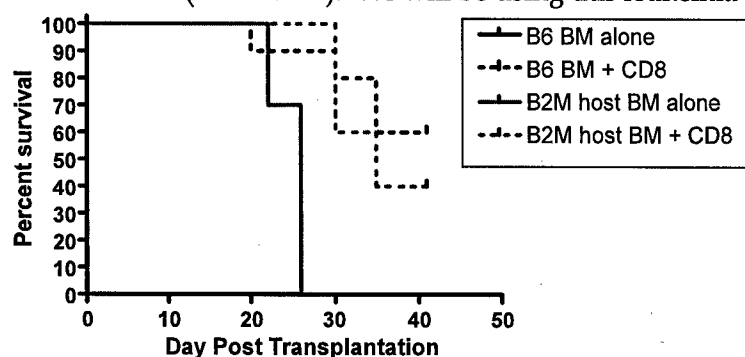


Figure 1. Radiation-resistant host APCs are not required for CD8-mediated GVL. B6 or B6 $\beta 2M^{-/-}$ mice were irradiated and reconstituted with T cell depleted C3H.SW BM, 15,000 B6 mBC-CML cells, with or without 4×10^6 C3H.SW CD8 cells. Survival in $\beta 2M^{-/-}$ and B6 recipients of CD8 cells were not significantly different whereas $\beta 2M^{-/-}$ hosts that received CD8 cells had superior survival as compared to $\beta 2M^{-/-}$ hosts that did not receive CD8 cells..

4. Determine the roles of donor APCs in CD8 GVL.

After obtaining the results in A.1., we chose to wait until we had sorted BC-CML cells available. The donor C3H.SW $\beta 2M^{-/-}$ mice have already been bred for this experiment which will be performed next month.

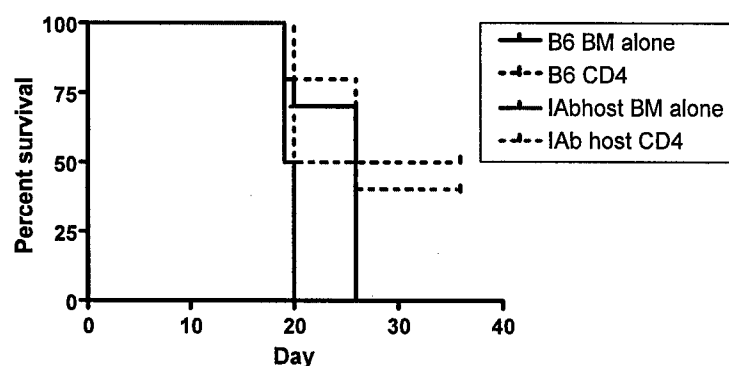


Figure 2. Radiation-resistant host APCs are not required for CD4-mediated GVL. B6 or B6 IAb^{-/-} mice were irradiated and reconstituted with T cell depleted C3H.SW BM, 10,000 B6 mBC-CML cells, with or without 4×10^6 C3H.SW CD4 cells. Survival in IAb^{-/-} and B6 recipients of CD4 cells were not significantly different whereas IAb^{-/-} hosts that received CD4 cells had superior survival as compared to $\beta 2M^{-/-}$ hosts that did not receive CD4 cells.

5. Determine roles of recipient APCs, CD4 GVL.

To do so we used B6IA^{b/-} (MHCII^{-/-}) mice as recipients in a GVL experiment using C3H.SW (H-2^b) donors.

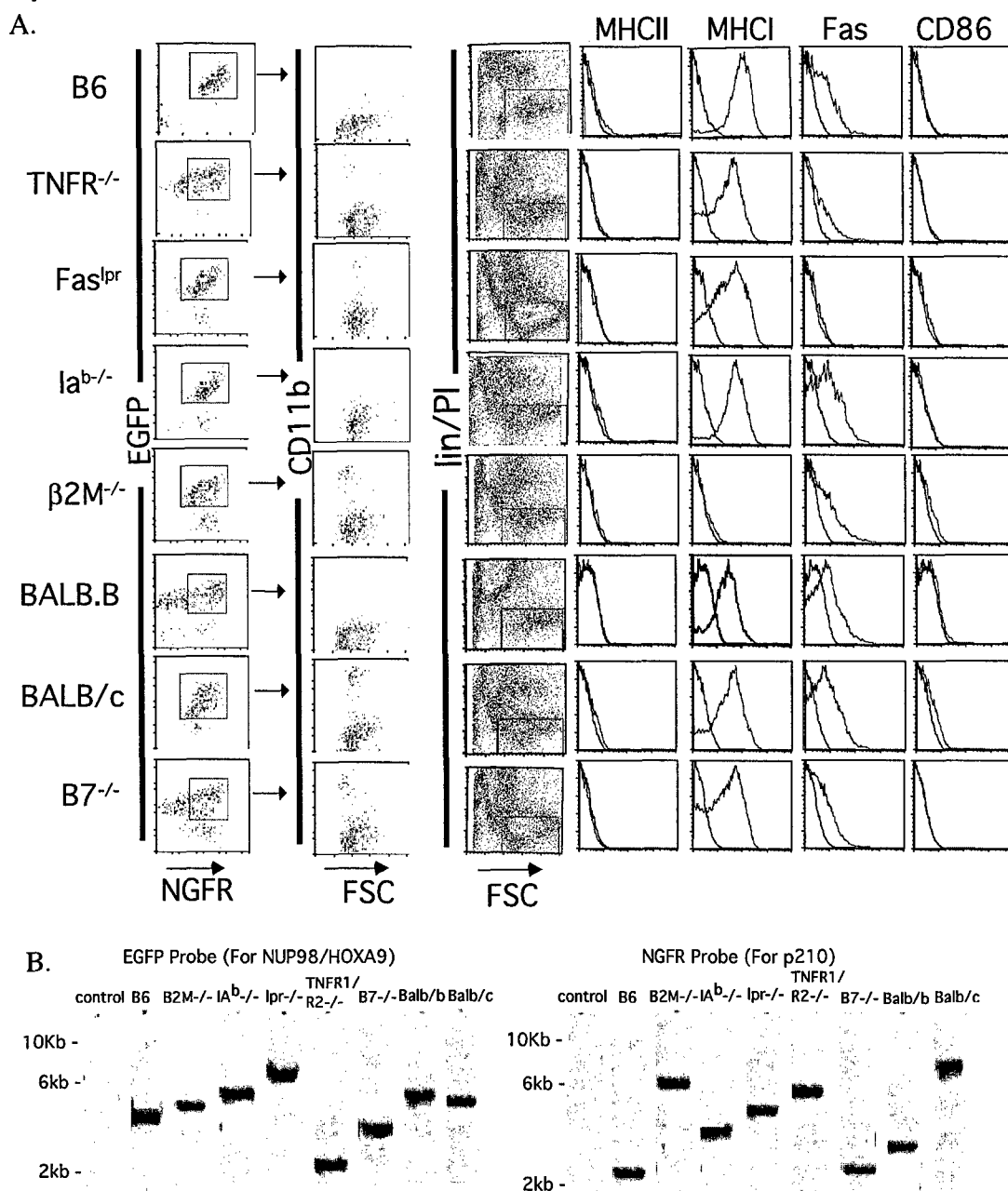


Figure 3. Generation of mBC-CML in 8 strains. Mice received 5-fluorouracil (5-FU) and BM cells were harvested 4 days later. Cells underwent spin infection with NH and p210 retrovirus at a 50:1 ratio. We used a low titer of p210/NGFR to minimize infection with this virus but not the NH virus. As controls, BM was infected with only NH or p210. Infected cells were injected into sublethally irradiated mice. Mice developed mBC-CML and spleens were harvested individually and single cell suspensions frozen. 10^6 cells were then injected into sublethally irradiated secondary recipients. **A.** Shown are FACS of representative spleens. Cells are NGFR⁺EGFP⁺ with few of these expressing CD11b (first 2 columns). Frozen cells were thawed and stained with a lineage cocktail of biotin conjugated antibodies (CD11b, Gr-1, CD4, CD8, TERR119, B220) and streptavidin PerCP. Propidium iodide was added to exclude dead cells. After gating on live lineage⁻ cells (third column), all cells were EGFP⁺NGFR⁺ and vice versa (not shown). Shown is expression of MHCII, MHCI, Fas and CD86. Note the absence of MHCI and Fas in $\beta 2M^{-/-}$ and Fas^{lpr} mBC-CML respectively. CD86 expression was not seen on any isolates. **B.** Southern blot of mBC-CML clones. After tertiary transfers spleens from leukemic mice were isolated and frozen in aliquots. An aliquot was thawed and genomic DNA was isolated, digested with EcoR1 and southern blotted. The blots were probed with an EGFP probe (left panel) and an NGFR probe (right panel) to determine clonality for insertion of the NUP98 and p210 expressing retrovirus, respectively. EcoR1 cuts only once in the vector, so each fragment represents a portion of the vector and the genomic DNA into which it integrated.

CD4-mediated GVL was significant in both wt and $IA^{b-/-}$ recipients ($P=0.03$), but survival was not significantly different in the two CD4 recipient groups ($P=0.37$) (Fig. 2). A repeat experiment is planned with sorted mBC-CML cells.

6. Induce mBC-CML in MHCII^{-/-}, MHCII^{-/-} and B71/B72^{-/-} mice. This task has been accomplished. The phenotype and the in vivo leukemia-induction capacity of the KO cells is shown in Fig. 3. Clonality is demonstrated by southern blot analysis in Fig. 4). These cells have also been compared to wild type cells for their ability to induce clinical leukemia and they are comparable (Fig. 3).

B. Aim 2 Tasks.

1. Establish mBC-CML in $\beta 2M^{-/-}$ and MHCII^{-/-} (IA^{b/-}) mice.

This has been accomplished and in addition these cells have been determined to be clonal by southern blot analysis (Fig 3 and Fig. 4).

2. Test GVL against MHCII and MHCII deficient mBC-CML. To do so we compared GVL against wild type and these gene deficient leukemias. Both of these experiments are ongoing. CD8-mediated GVL (using the C3H.SW→B6 strain pairing) required direct cognate interactions as survival in recipients of $\beta 2M^{-/-}$ mBC-CML with and without donor CD8 cells was equivalent ($P=0.08$) and no CD8 recipients survived. Survival of recipients of wt BC-CML and CD8 cells was superior to recipients of $\beta 2M^{-/-}$ BC-CML and CD8 cells ($P=0.0011$; Fig. 5). Similarly, GVL is thus far reduced against MHCII^{-/-} BC-CML (Fig. 6). However, we cannot yet exclude that some aspect of CD4-mediated GVL is independent of cognate CD4⁺ T cell:BC-CML contacts.

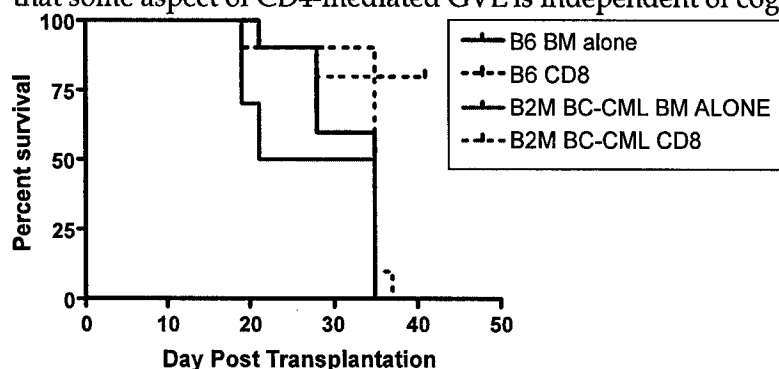


Figure 4. CD8-mediated GVL requires cognate interactions. Recipient B6 mice were irradiated and reconstituted with T cell depleted C3H.SW BM, 15,000 B6 or B6 $\beta 2M^{-/-}$ mBC-CML cells, with or without 4×10^6 C3H.SW CD8 cells. All mice received anti-NK1.1 on days -2 and -1 to prevent NK cell-mediated killing of MHCII^{-/-} mBC-CML. Survival curves of recipients of $\beta 2M^{-/-}$ mBC-CML mice that did or did not receive CD8 cells were not significantly different and all mice in these groups died from leukemia.

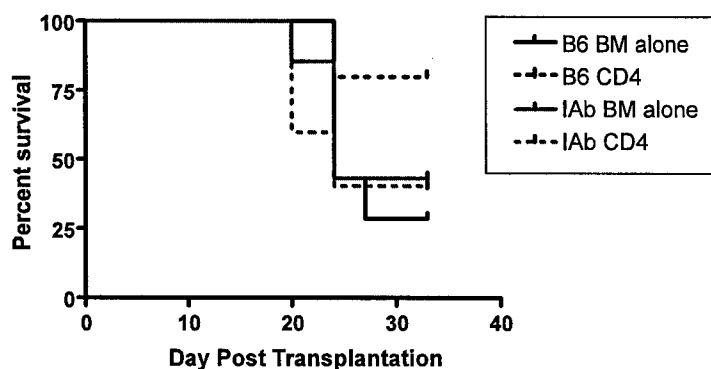


Figure 5. Maximal CD4-mediated GVL requires cognate interactions. Recipient B6 mice were irradiated and reconstituted with T cell depleted C3H.SW BM, 10,000 B6 or B6 IA^{b/-} mBC-CML cells, with or without 4×10^6 C3H.SW CD4 cells. Survival curves of recipients of IA^{b/-} mBC-CML mice that did or did not receive CD4 cells were not significantly different. This experiment is ongoing.

3. Establish mBC-CML in TNFR1/TNFR2^{-/-}, betac^{-/-}, Fas^{-/-}, IFN- γ receptor^{-/-} mice.

We have thus far established leukemias in TNFR1/TNFR2^{-/-} and Fas^{-/-} cells (Fig. 3). We have obtained IFN- γ R^{-/-} mice on a B6 background and will be crossing these to produce heterozygotes. We have not yet obtained betac^{-/-} mice.

4. Test GVL against gene deficient leukemias in B.3. Experiments testing both CD4 and CD8-mediated GVL against TNFR1/TNFR2^{-/-} and Fas^{-/-} leukemias were begun on 10/13 and 9/28 respectively.

C. Additional Data and New Plans.

1. mBC-CML cells express high levels of B7-H1. B7-H1 is a B7-family member that is a ligand for the receptor PD-1, expressed on activated T cells. PD-1 inhibits T cell activation and function and has been implicated in resistance to T cell-mediated anti-tumor responses. We analyzed mBC-CML cells by FACS and observed high level expression of B7-H1 in contrast to much lower expression of B7-DC. Murine chronic phase CML cells had variable and similar expression of B7-H1 and B7-DC. We have obtained B7-H1 and B7-DC gene deficient mice from

Lieping Chen and Drew Pardoll. These mice are breeding in our colony and we plan to make mBC-CML cells using these mice to test the hypothesis that B7-H1 or B7-DC contribute to GVL resistance.

2. Generation of mBC-CML cells that do not express TGF- β or overexpress TGF- β . Another potential mechanism for GVL resistance could be leukemic expression of TGF- β . In collaboration with Richard Flavell we plan to test this hypothesis by creating leukemic cells that either overexpress TGF- β or do not express it at all. To do so we have created a retrovirus that expresses p210 and cre recombinase and another retrovirus that expresses p210 and TGF- β 1. The cre virus will be used to create BC-CML cells that do not express TGF- β 1. This will be

done by infecting mice homozygous for a floxed TGF- β 1 locus. We are also crossing the ROSA-ER-cre mice, which carry an express a tamoxifen-inducible cre expression cassette to the TGF- β 1 floxed mice. We will then create mBC-CML in these mice and can generate a TGF- β null mouse by treating the cells with tamoxifen.

3. We are obtaining TRAIL receptor (TRAIL-R) deficient mice from Astar Winoto at UCSF. Trail has been implicated as an important killing mechanism for GVL. However, this has been established through the use of TRAIL-/- T cells. T cell activation and maturation into memory cells may be impaired in these mice and therefore the result could have been do to this rather than due to TRAIL as an actual end effector mechanism. Thus the use of TRAIL-R deficient leukemias would be a better approach.

III. Key Results.

A. Establishment of clonal gene deficient leukemias.

B. Host APCs are not required for CD8 or CD4-mediated GVL.

C. Cognate interactions are required for CD8-mediated GVL.

D. Cognate interactions are important and may be required for CD4-mediated GVL.

E. mBC-CML cells express high levels of B7-H1.

IV. Reportable outcomes. We are close to having sufficient data to report on the effector mechanisms of GVL and on APC requirements.

V. Conclusions

We now have nearly all of the necessary reagents to completely accomplish our Aims by the end of this funding year. In addition, we have established new directions to further explore the GVL resistance of BC-CML.

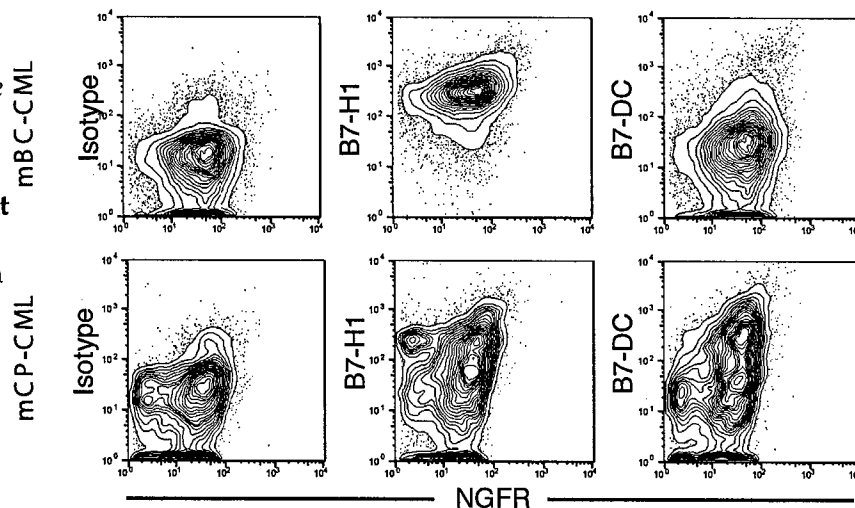


Figure 6. mBC-CML cells express high levels of B7-H1. mBC-CML (first row) and mCP-CML cells were stained with antibodies against NGFR and B7-H1 (second column), B7-DC (third column) or an isotype control (first column). Note uniform high level of staining for B7-H1 in mBC-CML cells.